INHIBITOR DEVELOPMENT FOR 4-HYDROXYPHENYLPYRUVATE DIOXYGENASE, EMPLOYING TYROSINEMIA I AS A MODEL FOR HUMAN DISEASES MEDIATED BY 2-OXOACID UTILIZING DIOXYGENASES

FIELD OF THE INVENTION

[0001]   The present invention is directed to methods of inhibiting 4-hydroxyphenylpyruvate dioxygenase in a living system, treating a patient with tyrosinemia, and regulating plant growth.

BACKGROUND OF THE INVENTION

[0003]   Tyrosinemia I, a recessively inherited disease that profoundly disrupts liver and kidney function, is caused by the deficiency of fumarylacetoacetate hydrolase (EC 3.7.1.2). The pathogenesis of potentially lethal liver damage in tyrosinemia I involves, however, two additional enzymes: 4-hydroxyphenylpyruvate dioxygenase (4-HPPD; EC 1.13.11.27), which forms the actual hepatotoxins; and prolyl 4-hydroxylase (P-4H; E.C. 1.14.11.2), required for the reactive hepatic fibrosis and indispensable for collagen synthesis in general. 4-HPPD and P4-H are non-heme iron enzymes, and each consumes one molecule of atmospheric oxygen and one 2-oxoacid moiety while hydroxylating one carbon atom. 4-HPPD and P-4H have long been classified together as 2-oxoacid-utilizing dioxygenases (Abbott et al., "Alpha-Ketoglutarate-Coupled Dioxygenases," in Hayaishi, ed. Molecular Mechanisms of Oxygen Activation, New York: Academic Press, pp. 167-214 (1974), and, therefore, tyrosinemia I ranks as a model for human diseases mediated by this class of enzymes, diseases that prominently include all fibrotic conditions. A single, collective catalytic pathway – the 'HAG' mechanism, proposed in 1982 by Hanauske-Abel and Günzler – resolves the substrate alignment, catalytic orbital interactions, and product release of the 2-oxoacid-utilizing dioxygenases (Hanauske-Abel et al., "A Stereochemical Concept for the Catalytic Mechanism of Prolyl Hydroxylase. Applicability to Classification and Design of Inhibitors," J. Theor. Biol. 94:421-55 (1982); Hanauske-

Abel, "Über einen Stereochemischen Vorschlag für den katalytischen Mechanismus
der Prolylhydroxylase, seine Anwendung zur Klassifizierung und Formulierung von
Hemmstoffen sowie die Entwicklung und Erprobung eines 'maßgeschneiderten'
neuartigen Inhibitors," Department of Pharmacol. Toxicol., Philipps University,

5　　Marburg an der Lahn, Germany, M.D., Ph.D., Thesis (1983); Hanauske-Abel,
"Fibrosis: Representative Molecular Elements, A Basic Concept, and Emerging
Targets for Suppressive Treatment," in Zakim, eds. Hepatology, A Textbook of Liver
Disease," Philadelphia: W.B. Saunders, pp. 465-506 (1996), and has been extremely
useful for the rational, often computer-assisted discovery of inhibitors and candidate

10　　drugs targeting these enzymes (Hanauske-Abel et al., "The HAG Mechanism: A
Molecular Rationale for the Therapeutic Application of Iron Chelators in Human
Diseases Involving the 2-Oxoacid Utilizing Dioxygenases," Curr. Med. Chem.
10:1005-19 (2003); Hanauske-Abel, "Prolyl 4-Hydroxylase, A Target Enzyme for
Drug Development. Design of Suppressive Agents and the In Vitro Effects of

15　　Inhibitors and Proinhibitors," J. Hepatol., 13(Suppl.3):S8-S16 (1991); Günzler et al.,
"Prolyl 4-Hydroxylase Inhibitors," in Guzman NA ed., Prolyl Hyroxylase, Protein
Disulfide Isomerase, and Other Structurally Related Proteins, New York: Decker,
pp. 65-96 (1997). As expected, the HAG mechanism-utilizing enzymes display a
remarkable cross-species susceptibility to such inhibitors; e.g., human and plant 4-

20　　HPPD are similarly sensitive to the cyclohexanetrione class of plant growth retardants
(Schulz et al., "SC-0051, A 2-Benzoyl-Cyclohexane-1,3-Dione Bleaching Herbicide,
is a Potent Inhibitor of the Enzyme P-Hydroxyphenylpyruvate Dioxygenase," FEBS
Lett. 318:162-166 (1993); Ellis et al., "Inhibition of 4-Hydroxyphenylpyruvate Di-
Oxygenase by 2-(2-Nitro-4-Trifluormethylbenzoyl)-Cyclohexane-1,3-Dione and by 2-

25　　(2-Chloro-4-Methanesulfonylbenzoyl)-Cyclohexane-1,3-Dione," Toxicol. Appl.
Pharmacol. 133:12-19 (1995). The cyclohexanetrione herbicides also inhibit other 2-
oxoacid-utilizing dioxygenases in plants, such as the hydroxylases essential for
synthesis of gibberellin hormones (Nakayama et al., "Effect of a Plant-Growth
Regulator, Prohexadione, on the Biosynthesis of Gibberellins in Cell-Free Systems

30　　Derived from Immature Seeds, Plant Cell Physiol. 31:1183-90 (1990); Rademacher,
"Biochemical Effects of Plant Growth Retardants," in Gausmann, ed., Plant
Biochemical Regulators, New York: Dekker, pp. 169-200 (1991). Like human P-4H,

- 3 -

the gibberellin hydroxylases use the 2-oxoacid moiety of 2-oxoglutarate (Hanauske-Abel, "Fibrosis: Representative Molecular Elements, A Basic Concept, and Emerging Targets for Suppressive Treatment," in Zakim, eds. Hepatology, A Textbook of Liver Disease," Philadelphia: W.B. Saunders, pp. 465-506 (1996).

5   [0004]   In children with tyrosinemia I, one of these herbicides is used therapeutically to inhibit 4-HPPD, thus suppressing formation of the hepatotoxins maleylacetoacetate, fumarylacetoacetate, and their saturated derivatives (Lindstedt et al., "Treatment of Hereditary Tyrosinemia Type I by Inhibition of 4-Hydroxyphenylpyruvate Dioxygenase," Lancet 340:813-17 (1992)). Introduced in

10   1992, this agent, 2-2(-nitro-4-trifluoromethylbenzoyl)-cyclohexane-1,3-dione or NTBC (Orfadin™; Swedish Orphan International AB, Harpenden/United Kingdom), has benefited several hundred tyrosinemia I patients and has changed at least the time frame for liver transplantation, presently still the definitive treatment (Wappner, "Disorders of Amino Acid and Organic Acid Metabolism," in McMillan, eds., Oski's

15   Pediatrics, Philadelphia: Lippincott, pp. 1828-1847 (1999); Holme et al., "Tyrosinemia Type I and NTBC [2-(2-Nitro-4-Trifluoromethylbenzoyl)-1,3-Cyclohexanedione]," J. Inherit Metab. Dis., 21:507-17 (1998). However, no specific consideration was given to any inhibitory effect NTBC might exert, or might fail to exert, on other 2-oxoacid-utilizing dioxygenases in humans, and its mode of binding

20   to 4-HPPD, the basis for its clinical utility, also has received little attention.

[0005]   The present invention is directed to overcoming these deficiencies in the art.
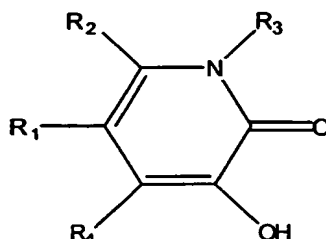
SUMMARY OF THE INVENTION

25

[0006]   The present invention is directed to a method of inhibiting 4-hydroxyphenylpyruvate dioxygenase in a living system by administering to the living system an effective amount of a compound of formulas I or II or III or derivatives thereof as follows:
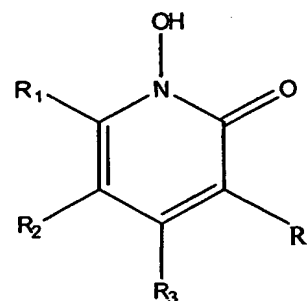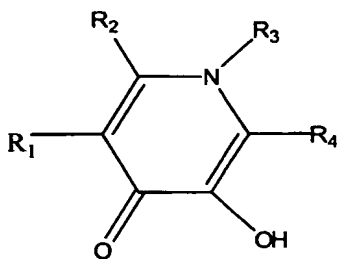
30

- 4 -
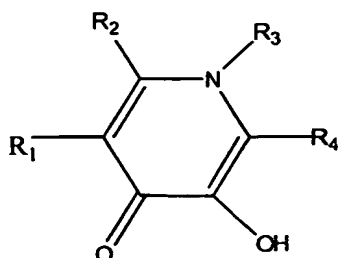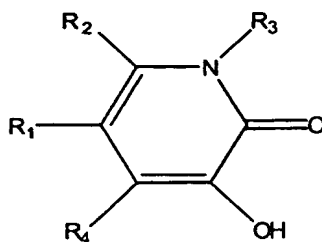

I   II   III

R^1, R^2, R^3, and R^4 each individually represent a hydrogen, an alkyl, alkenyl, or alkoxy group containing 1 to about 8 carbon atoms, an aryl, aralkyl, or cycloalkyl group

5   containing about 5 to 12 carbon atoms, or a carboalkoxy or carbamyl group containing up to 8 carbon atoms, or a peptide or peptidomimetic moiety containing 10 to about 30 carbon atoms under conditions effective to inhibit 4-hydroxyphenylpyruvate dioxygenase in the living system.
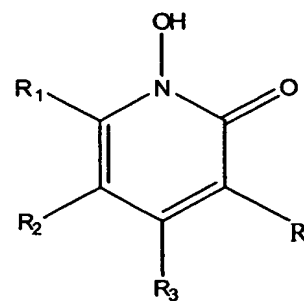
[0007]   Another aspect of the present invention is directed to a method of

10   treating a patient with tyrosinemia by administering to the patient an effective amount of a compound of formulas I or II or III or derivatives thereof as follows:

I   II   III

R^1, R^2, R^3, and R^4 each individually represent a hydrogen, an alkyl, alkenyl, or alkoxy

15   group containing 1 to about 8 carbon atoms, an aryl, aralkyl, or cycloalkyl group containing about 5 to 12 carbon atoms, or a carboalkoxy or carbamyl group containing up to 8 carbon atoms, or a peptide or peptidomimetic moiety containing 10 to about 30 carbon atoms under conditions effective to treat tyrosinemia.

[0008]   Yet another aspect of the present invention relates to a method of

20   regulating plant growth by administering to a plant an effective amount of a compound of formulas I or II or III or derivatives thereof as follows:

- 5 -

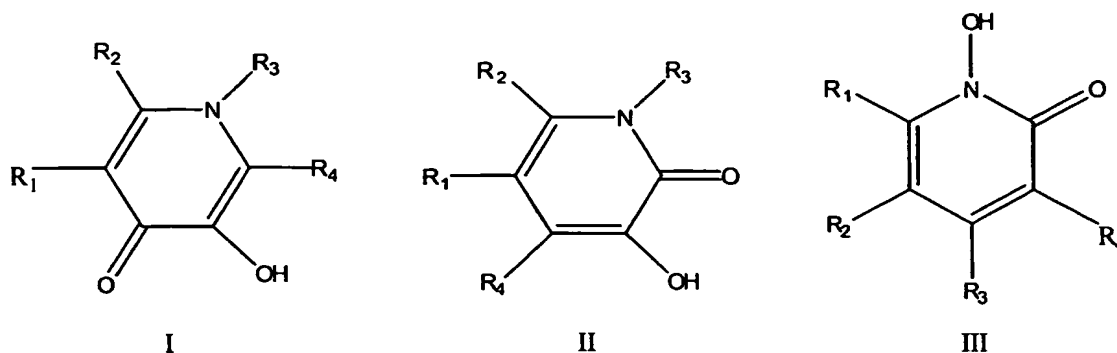I   II   III

R^1, R^2, R^3, and R^4 each individually represent a hydrogen, an alkyl, alkenyl, or alkoxy group containing 1 to about 8 carbon atoms, an aryl, aralkyl, or cycloalkyl group

5   containing about 5 to 12 carbon atoms, or a carboalkoxy or carbamyl group containing up to 8 carbon atoms, or a peptide or peptidomimetic moiety containing 10 to about 30 carbon atoms under conditions effective to regulate plant growth.

[0009]   Applicants have discovered that treatment of tyrosinemia I with NTBC allows the simultaneous assessment of inhibition of 4-HPPD and P4-H *in vivo.*

10   Surrogate biomarkers for the activity of either enzyme in a patient with established tyrosinemia I are monitored and prospectively measured for the first 100 days of NTBC administration. Only the 4-HPPD biomarkers normalized. These empirical observations are due to a general biologic factor—i.e., the distinct difference between the active site topography of 4-HPPD and of P4-H.

15

BRIEF DESCRIPTION OF THE DRAWINGS

[0010]   Figure 1 shows the effect of NTBC. The shaded area indicates the range of normals, the hatched bar the NTBC treatment period. Biomarkers for 4-

20   HPPD activity: Plasma succinylacetone (large open circles; normal: <0.1 μmol/L) and urine succinylacetoacetate (small open circles; normal: < 1.0 mmol/mol creatinine). Biomarkers for P4-H activity; PIIINP, indicating collagen type III (solid squares; normal: <0.8 U/ml); NC1, indicating collagen type IV (solid circles; normal: < 9.5 ng/ml); and NTD, indicating collagen type VI (solid triangles; normal: < 530

25   ng/ml). Control biomarker for P4-H activity: P1, derived from laminin (open triangles; normal: <1.8 ng/ml).

- 6 -

[0011]   Figures 2A-B show the structure and binding of NTBC. Figure 2A
shows the structure of NTBC. The domains shared between 4-
hydroxyphenylpyruvate (4-HPP), the substrate of 4-HPPD, and the drug 2-(2-nitro-4-
trifluoromethylbenzoyl)-cyclohexane-1,3-dione (NTBC) are highlighted (I, benzene

5   ring; II, 2-oxoacid group or its analog). NTBC displays an acetylacetone moiety that
preferentially tautomerizes into its keto-enol form (Rademacher, "Biochemical
Effects of Plant Growth Retardants," in Gausmann, ed., Plant Biochemical
Regulators, New York: Dekker, pp. 169-200 (1991), which are hereby incorporated
by reference in their entirety), enabling interaction with the non-heme iron atom (Fe)

10   at the active site of 4-HPPD, 4-HPP binds via bidentate, NTBC via terdentate
coordination. Figure 2B shows the binding of NTBC to 4-HPPD. The steric
arrangement of the ligands NTBC (4) and 4-HPP (3) at the non-heme iron atom (1) in
the active site pocket of 4-HPPD is depicted. The peptide backbone of the enzyme,
shown as gray ribbon, has been cut open for visualization. Two histidine (5a) and one

15   carboxylate (5b) side chain coordinate the iron atom to the apoprotein. This non-heme
iron atom displays the typical 'bipolar', cisoid alignment of reactants as required by
the HAG mechanism (Hanauske-Abel et al., "A Stereochemical Concept for the
Catalytic Mechanism of Prolyl Hydroxylase. Applicability to Classification and
Design of Inhibitors," J. Theor. Biol. 94:421-55 (1982); Hanauske-Abel et al., "The

20   HAG Mechanism: A Molecular Rationale for the Therapeutic Application of Iron
Chelators in Human Diseases Involving the 2-Oxoacid Utilizing Dioxygenases," Curr.
Med. Chem. 10:1005-19 (2003); and Hanauske-Abel, "Fibrosis: Representative
Molecular Elements, A Basic Concept, and Emerging Targets for Suppressive
Treatment," in Zakim, eds. Hepatology, A Textbook of Liver Disease," Philadelphia:

25   W.B. Saunders, pp. 465-506 (1996), which are hereby incorporated by reference in
their entirety): apoenzyme residues on one half-sphere, and substrates or inhibitors on
the other half-sphere. The O_2-derived dioxygen substrate is shown broken open in
eggshell fashion (2) to display the position occupied by the nitro group of NTBC.
The appenzyme residues that hold the non-heme iron atom, identified for bacterial

30   and, in italics, for human 4-HPPD, occur in highly conserved segments (73%
identity/similarity by ClustalW formatted alignment of the bacterial and human
sequences: accession numbers of 1CJXA and AAC73008, respectively). Note that in
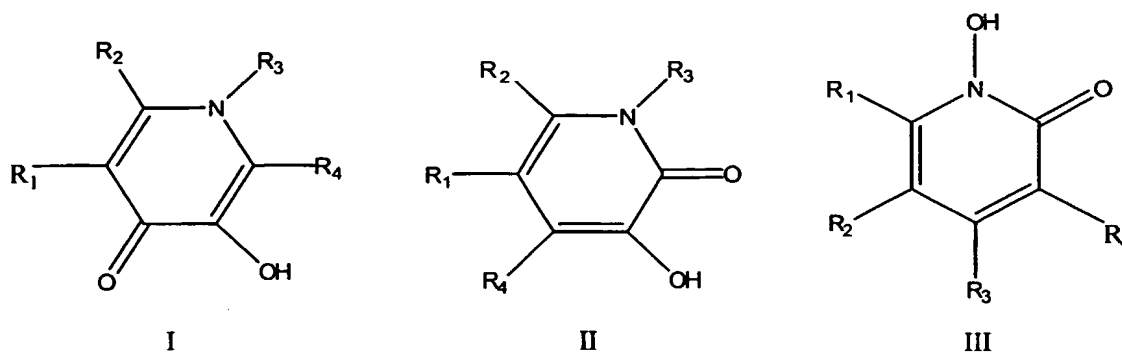
- 7 -

NTBC as well as in the substrate 4-HPP, the planes of the shared domains (I. and II.)

stand almost perpendicular on each other.

[0012]   Figure 3 shows the binding of 1,2-dimethyl-3-hydroxypyrid-4-one

(DMHP), a representative compound of the present invention and also a known

5   inhibitor of prolyl 4-hydroxylase and thus collagen biosynthesis (United States Patent

Nos. 5,789,426; 5,965,585; and 5,965,586, which are hereby incorporated by

reference in their entirety) to the active site of 4-HPPD. The steric arrangement of the

ligands, either the substrate 4-HPP (3) or the inhibitor DMPH (4), at the non-heme

iron atom (1) in the active site pocket of 4-HPPD is depicted. The iron-bound

10   dioxygen is highlighted (2), as are the residues involved in binding of the metal atom

(5a and 5b) or of the substrate (5c). Thus, in contrast to NTBC, DMHP can access and

block the active site of prolyl 4-hydroxylase as well as of 4-HPPD.

DETAILED DESCRIPTION OF THE INVENTION

15

[0013]   The present invention is directed to a method of inhibiting 4-

hydroxyphenylpyruvate dioxygenase in a living system by administering to the living

system an effective amount of a compound of formulas I or II or III or derivatives

thereof as follows:

20

I   II   III

R^1, R^2, R^3, and R^4 each individually represent a hydrogen, an alkyl, alkenyl, or alkoxy

group containing 1 to about 8 carbon atoms, an aryl, aralkyl, or cycloalkyl group

containing about 5 to 12 carbon atoms, or a carboalkoxy or carbamyl group

containing up to 8 carbon atoms, or a peptide or peptidomimetic moiety containing 10

- 8 -

to about 30 carbon atoms under conditions effective to inhibit 4-
hydroxyphenylpyruvate dioxygenase in the living system.

[0014]   The alkyl, alkenyl, alkoxy, aryl, aralkyl, and cycloalkyl groups
represented by R_1, R_2, R_3, and R_4 can be substituted or unsubstituted. Examples of
5   unsubstituted alkyl groups are methyl, ethyl, propyl, isopropyl, butyl, sec-butyl,
pentyl, hexyl, and the like. Unsubstituted alkenyl groups can be 2-propenyl, 2-
butenyl, 3-butenyl, 2-methyl-2-butenyl, and the like. Unsubstituted alkoxy groups
can be methoxy, ethoxy, propoxy, sopropoxy, and the like. Unsubstituted aryl groups
can be phenyl or naphthyl. Aralkyl groups can be, for example, benzyl and
10   phenylethyl. Cycloalkyl groups can be cyclopentyl, cyclohexyl, 4-methyl cyclohexyl,
and the like. For substituted alkyl, alkenyl, alkoxy, aryl, aralkyl, and cycloalkyl
groups, substituents can include, for example, halo, alkoxy, amino, hydroxy, carboxy,
carboalkoxy, and carbamyl. Aryl and aralkyl groups can, in addition, contain alkyl
substituents.

15   [0015]   Table I contains representative forms of the hydroxypyridone
compounds of Formula (I) of the present invention:

Table I

	Trivial name	R_1	R_2	R_3	R_4
I	L-mimosine	$-CH_2CH(COOH)NH_2$	H	H	H
	HK-1, CP20, L1, DMHP	$-CH_3$	$-CH_3$	H	H
	HK-2, CP94	$-CH_2CH_3$	$-CH_2CH_3$	H	H
	CP93	$-CH_3$	$-CH_2CH_3$	H	H
	CP96	$-(CH_2)_2OCH_3$	$-CH_2CH_3$	H	H
	HK-26, CP21	$-CH_2CH_3$	$-CH_3$	H	H
	HK-27, CP22	$-(CH_2)_2CH_3$	$-CH_3$	H	H
	HK-16	$-CH_2CH=CH_2$	$-CH_3$	H	H
	CP23	$-CH_2(CH_3)_2$	$-CH_3$	H	H
	CP40	$-(CH_2)_2OH$	$-CH_3$	H	H
	CP41	$-(CH_2)_3OH$	$-CH_3$	H	H
	CP42	$-(CH_2)_4OH$	$-CH_3$	H	H
	CP43	$-(CH_2)_5OH$	$-CH_3$	H	H
	CP44	$-(CH_2)_2NH_2$	$-CH_3$	H	H
	HK-15, CP51	$-(CH_2)_2OCH_3$	$-CH_3$	H	H
	CP54	$-CH(CH_3)CH_2OCH_3$	$-CH_3$	H	H
	CP52	$-(CH_3)_3OCH_2CH_3$	$-CH_3$	H	H
		$-CH_3$	H	$-CH_3$	H
		$-CH_3$	H	H	$-CH_3$
		$-CH_2CH(COOC_2H_5)NH_2$	H	H	H

[0016]   Table II contains representative structures of the hydroxypyridone
compounds of Formula (II) of the present invention:

Table II

|	R_1	R_2	R_3	R_4
II	$-CH_3$	$-CH_3$	H	H
	$-CH_2CH_3$	$-CH_2CH_3$	H	H
	$-CH_3$	$-CH_2CH_3$	H	H
	$-(CH_2)_2OCH_3$	$-CH_2CH_3$	H	H
	$-CH_2CH_3$	$-CH_3$	H	H
	$-(CH_2)_2CH_3$	$-CH_3$	H	H
	$-CH_3$	H	$-CH_3$	H
	$-CH_3$	H	H	$-CH_3$

Compounds of Formula (I)

[0017]   Compounds of Formula (I) are synthesized by one of several general procedures.

Method A

[0018]   This method is adapted from Kontoghiorghes and Sheppard, Inorg. Chim. Acta 136:L11-L12 (1987), which is hereby incorporated by reference in its entirety. In brief, a 3-hydroxy-4-pyrone is refluxed for approximately six hours with three equivalents of a primary amine dissolved in an appropriate solvent. The reaction mixture is decolorized with charcoal, filtered, and the filtrate evaporated to give a dark residue. The residue is recrystallized from one to three times from an appropriate solvent to yield a solid product with a narrow melting point and an NMR spectrum consistent with the structure anticipated.
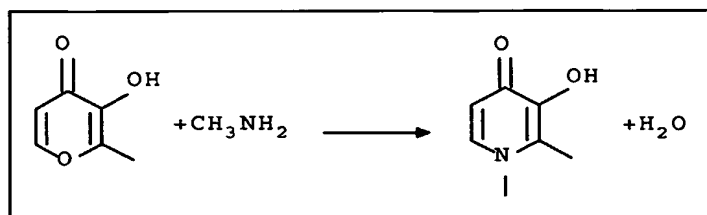
[0019]   In particular, 3-hydroxy-2-methyl-4-pyrone (10 g) was refluxed for 6.5 hours with three equivalents of aqueous methylamine (40%) in 200 ml of water. The reaction mixture was allowed to cool after which decolorizing charcoal was added to the solution, and the mixture was stirred for 0.5 hours. After filtration, the solvent was evaporated under reduced pressure, and the solid residue recrystallized three times from water to yield 1,2-dimethyl-3-hydroxypyrid-4-one (HK-1, L1, CP20, DMHP) as fine white needles.

[0020]   In a slight modification of this procedure, N-carboxymethyl-3-hydroxy-2-methylpyrid-4-one is prepared as described by Zhang et al., Can. J. Chem. 70:763-770 (1992), which is hereby incorporated by reference in its entirety. One equivalent of 3-hydroxy-2-methyl-4-pyrone and two equivalents of glycine are

dissolved in hot distilled water, the pH is adjusted to approximately 9 with 8 N

sodium hydroxide, and the reaction mixture is heated under reflux for 20 hours. After

cooling and decolorizing with charcoal, approximately half of the solvent is removed

under vacuum and 6 N hydrochloric acid is added to reduce the pH to approximately

5   3. A yellow solid precipitates which yields the product as off-white crystals after two

recrystallizations from water (mp 258-260C).

Method B

[0021]   This method is adapted from GB Patent No. 2,118,176A to Hider et al.,

10   which is hereby incorporated by reference in its entirety. In brief, a 3-hydroxy-4-

pyrone is converted to the corresponding 3-benzyloxy-4-pyrone via reaction with

benzyl chloride. A methanolic solution of the pyrone is added to an aqueous solution

of sodium hydroxide after which benzyl chloride is added and the reaction mixture

refluxed for approximately six hours. The solvent is evaporated under reduced

15   pressure, water is added, and then the product is extracted into an appropriate organic

solvent. After washing, the extract is dried over anhydrous magnesium sulfate and

the solvent evaporated to yield the crude 3-benzyloxy derivative which is used in the

next step without further purification. To a solution of the 3-benzyloxy compound in

an appropriate solvent is added a slight excess of primary amine. The reaction

20   mixture is stirred at room temperature for approximately six days after which it is

acidified to pH 2 with concentrated hydrochloric acid and evaporated to dryness. The

residue is washed with water and extracted into an appropriate organic solvent which

is then dried over magnesium sulfate and evaporated to dryness. To the residue is

added hydrobromic acid. This reaction mixture is heated on a steam bath for 30

25   minutes and then recrystallized from water to yield the N-substituted 3-hydroxypyrid-

4-one. The product melts sharply and has an NMR spectrum consistent with the

desired product. This synthesis is depicted as follows:
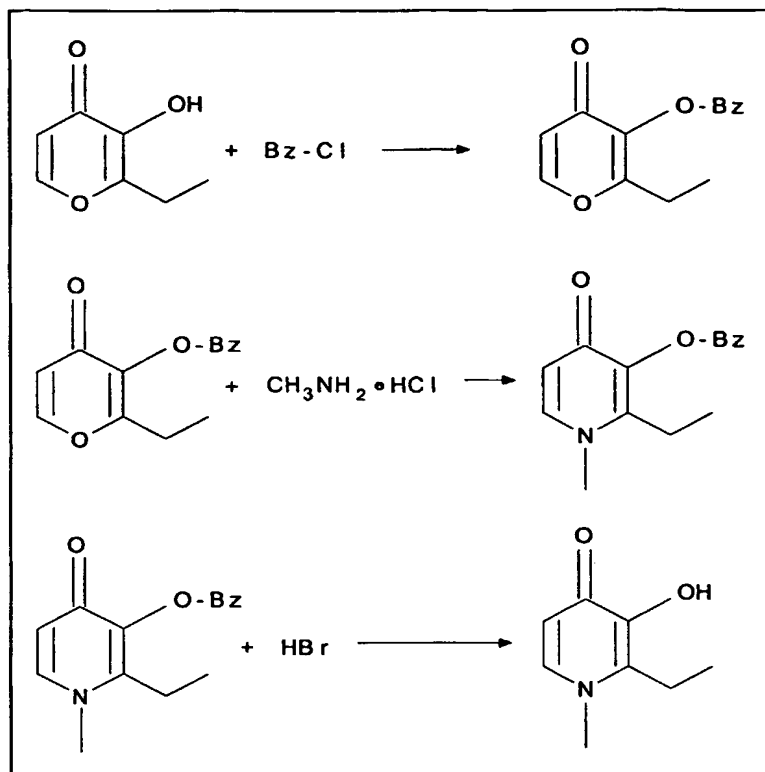
[0022]   In particular, 2-ethyl-3-hydroxy-4-pyrone (24.7 g) in 225 ml of
methanol is added to 25 ml of water containing 7.5 g of sodium hydroxide. To this
5   solution is added benzyl chloride (25.5 g) and the mixture is then refluxed for six
hours. Upon cooling, the solvent is removed under reduced pressure. The residue is
treated with 50 ml of water and then extracted three times with 25-ml aliquots of
dichloromethane. The combined extracts are washed twice with 5% (w/v) sodium
hydroxide, then twice with 25 ml of water and dried over magnesium sulfate.
10   Evaporation of the solvent yields crude 3-benzyloxy-2-ethyl-4-pyrone. This crude
pyrone (24.4 g) and 1.56 g of methylamine hydrochloride are then dissolved in 300 ml
of aqueous ethanol (100 ml) containing 2 g of sodium hydroxide. The solution is
stirred at room temperature for six days, acidified to pH 2 with concentrated
hydrochloric acid, and then evaporated to dryness. The residue is washed with water
15   and extracted twice into chloroform (50 ml). The combined extracts are dried over
anhydrous magnesium sulfate and evaporated to dryness yielding 3-benzyloxy-2-
ethyl-1-methylpyrid-4-one. To 2 g of this pyrid-4-one is added concentrated
hydrobromic acid (10 ml). The reaction mixture is heated on a steam bath for 30

- 13 -

minutes, and the product is recrystallized from water to yield 2-ethyl-3-hydroxy-1-
methylpyrid-4-one.

Method C

5   [0023]   This method is adapted from that of Bartulin et al., J. Heterocyclic
Chem. 29:1017-1019 (1992), which is hereby incorporated by reference in its entirety.
A 3-benzyloxy-4-pyrone, prepared as in Method B, is added to an ethanolic solution
of aqueous ammonia. The reaction mixture is stirred for approximately three days,
concentrated under reduced pressure, triturated with acetone, and the solid

10   recrystallized from ethanol to yield the corresponding 3-benzyloxypyrid-4-one. To a
solution of this pyrid-4-one in aqueous ethanol containing one equivalent of sodium
hydroxide was added an equivalent of n-alkyl bromide. The reaction mixture was
heated under reflux for 24 hours after which it was cooled, concentrated under
reduced pressure, and extracted with an appropriate solvent. After washing, the

15   organic phase with water, it is dried over magnesium sulfate. The product is obtained
upon concentration of the solution under reduced pressure. Crude 1-alkyl-3-
benzyloxypyrid-4-one in acetic acid containing 40% hydrobromic acid is then heated
on a steam bath for 30 minutes. The 1-alkyl-3-hydroxypyrid-4-one precipitates and is
subsequently recrystallized from benzene in good yield with a narrow melting point

20   and appropriate NMR spectrum. This is shown in the following synthesis:

+NH₄OH →

+CH₃(CH₂)₅Br →

+HBr / AcOH →
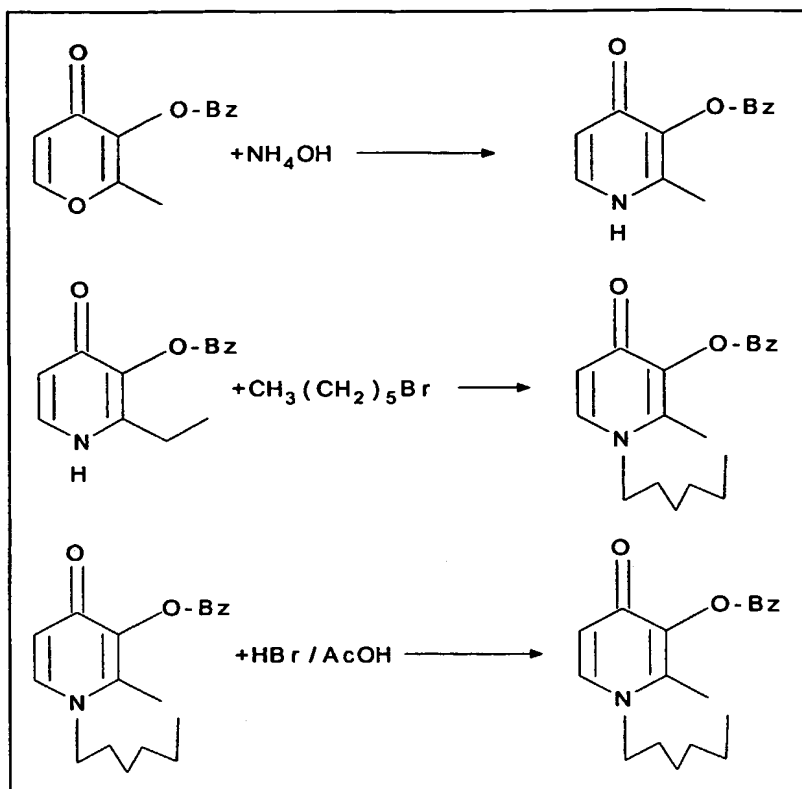
[0024] In particular, 3-benzyloxy-2-methyl-4-pyrone was prepared as
described in Method B. A solution containing 15.3 g of the pyrone, 160 ml of
aqueous ammonia (25%), and 80 ml of ethanol is stirred at room temperature for three
days. The solvent is removed under reduced pressure and some acetone is added.
The solid which precipitates is collected by filtration and recrystallized from ethanol
to yield (80%) 3-benzyloxy-2-methylpyrid-4-one with a melting point of 162-163C.
A solution containing 0.125 moles of the pyrid-4-one, 0.125 moles of n-hexyl
bromide, 0.125 moles of sodium hydroxide, 25 ml of water, and 200 ml of ethanol is
heated under reflux for 24 hours. After removal of the solvent under vacuum, the
residue is extracted with ethyl ether. The etherial solution is washed with water
yielding a precipitate which is crystallized from benzene after drying to give 3-
benzyloxy-1-hexyl-2-methylpyrid-4-one (95%, mp 46°C). A solution of this
compound in 80 ml of acetic acid containing 40% hydrobromic acid is then heated on
a steam bath for 30 minutes. The product is filtered off and crystallized from benzene
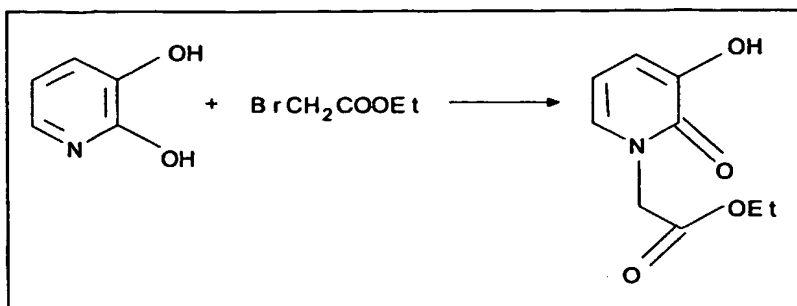to yield 1-hexyl-3-hydroxy-2-methylpyrid-4-one in 70% yield.

Compounds of Formula (II)

[0025]   Compounds of Formula (II) are synthesized by the general procedure
outlined in GB Patent No. 1,118,176A to Hider et al., which is hereby incorporated by
reference in its entirety. In brief, 2,3-dihydroxypyridine is mixed with an organic
halide in a sealed tube and heated at 140°C for 24 hours. The tube is then cooled in
an acetone/dry ice bath and opened. The excess halide is poured off, and water is
added to the dark residue. Sulfur dioxide gas is bubbled through the mixture until the
aqueous phase becomes clear. The pH of the reaction mixture is then adjusted to
approximately 6 with sodium carbonate, and the resulting solution is extracted with an
appropriate solvent after saturation with ammonium sulfate. The organic extracts are
dried over anhydrous sodium sulfate and concentrated under reduced pressure to yield
a solid which gives the desired N-substituted 3-hydroxypyrid-2-one after
crystallization from petroleum ether.
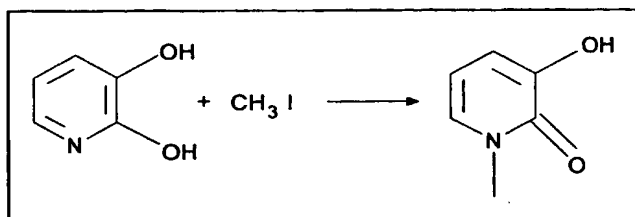
[0026]   In particular, 5.6 g of 2,3-dihydroxypyridine in 20 ml of methyl iodide
are heated in a sealed tube at 140°C for 24 hours. The tube is cooled in acetone/dry
ice, opened, and the excess methyl iodide poured off. Distilled water (10 ml) is
added, and the solution is treated with sulfur dioxide until clear. The pH of the
reaction mixture is adjusted to 6 with aqueous sodium carbonate (1 M) after which the
resulting solution is saturated with ammonium sulfate followed by extraction with
chloroform until the chloroform layer fails to give a blue color with aqueous ferric
chloride. The combined extracts are dried over sodium sulfate after which the solvent
is removed under reduced pressure and the residue crystallized from petroleum ether
to give 3-hydroxy-1-methylpyrid-2-one.

[0027] A related compound, 1-ethoxycarbonylmethyl-3-hydroxypyrid-2-one, is prepared by heating a mixture of 2,3-dihydroxypyridine (5 g) and 20 ml of ethylbromoacetate in a sealed tube at 140°C for 24 hours, as described by GB Patent No. 4,585,780 to Hider et al., which is hereby incorporated by reference in its entirety. After cooling in solid CO_2, the tube is opened, the reaction mixture poured off, and evaporated to dryness under vacuum to yield a yellow solid. Recrystallization from water yields the product as white crystals (5.4 g), MP 141-151°C.

Compounds of Formula (III)

[0028] Compounds of Formula (III) are synthesized by the general procedure outlined in Kellner et al., "Pharmacokinetics and Biotransformation of the Antimycotic Drugs Ciclopiroxolamine In Animals and Man After Topical and Systemic Administration," Arzneimittelforschung 31:1337-1353 (1981), which is hereby incorporated by reference in its entirety.

[0029] The compounds of the present invention can be administered topically or systemically to living beings. More particularly, in humans and animals, such administration can be orally; parenterally, i.e. by subcutaneous, intravascular, or intramuscular injection; intraperitoneally; intrathecally; or by topical application, e.g. to skin or eyes, or by application to the mucous membranes of the nose, throat, bronchial tree, or rectum, etc. They may be administered alone or with suitable pharmaceutical carriers, and can be in solid or liquid form such as tablets, capsules, powders, solutions, suspensions, or emulsions. The dosage of the active compound depends on the species of warm-blooded animal, the body weight, age, and mode of administration.

- 17 -

[0030]   The pharmaceutical products of the present invention are prepared by dissolving, mixing, granulating, or tablet-coating processes which are known *per se.*
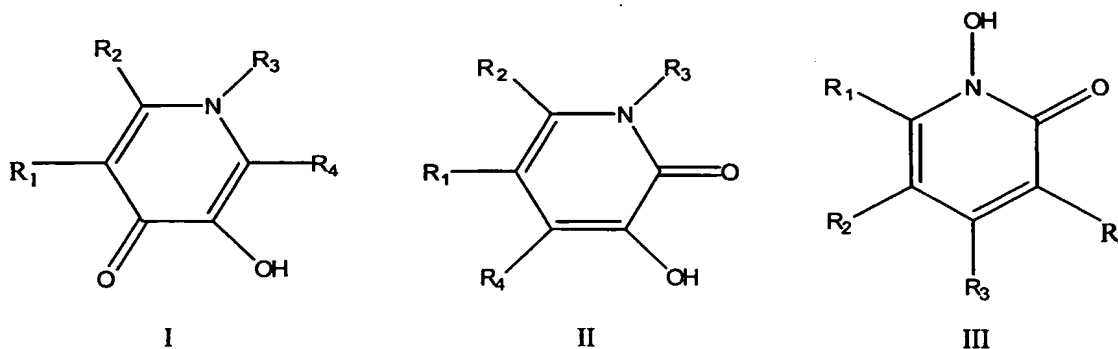
[0031]   For oral administration, the active compounds or their physiologically tolerated derivatives such as salts, esters, or amides, are mixed with the additives

5   customary for this purpose, such as vehicles, stabilizers, or inert diluents, and are converted by customary methods into a suitable form for administration, such as tablets, coated tablets, hard or soft gelatin capsules, aqueous, alcoholic, or oily suspensions, or aqueous, alcoholic or oily solutions. Examples of suitable inert vehicles are conventional tablet bases such as lactose, sucrose, or cornstarch in

10   combination with binders like acacia, cornstarch, gelatin, or with disintegrating agents such as cornstarch, potato starch, alginic acid, or with a lubricant like stearic acid or magnesium stearate. Examples of suitable oily vehicles or solvents are vegetable or animal oils, such as sunflower oil or fish-liver oil. Preparations can be effected both as dry and as wet granules.

15   [0032]   For parenteral administration (subcutaneous, intravascular, or intramuscular injection), the active compounds or their physiologically tolerated derivatives such as salts, esters, or amides, are converted into a solution, suspension, or emulsion, if desired, with the substances customary and suitable for this purpose, such as solubilizers or other auxiliaries. Examples are: sterile liquids such as water

20   and oils, with or without the addition of a surfactant and other pharmaceutically acceptable adjuvants. Illustrative oils are those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, or mineral oil. In general, water, saline, aqueous dextrose and related sugar solution, and glycols such as propylene glycol or polyethylene glycol, are preferred liquid carriers, particularly for

25   injectable solutions.

[0033]   For use as aerosols, such as required for the spraying of plants, the active compounds or their derivatives such as salts, esters, or amides, may be dissolved or suspended in an acceptable liquid and packaged in a pressurized aerosol container together with suitable propellants, for example, hydrocarbon propellants

30   like propane, butane, or isobutane with conventional adjuvants. The agents, in accordance with the present invention, may also be administered from a non-pressurized container such as a nebulizer or atomizer.

[0034]   For topical administration to external or internal body surfaces, e.g., in the form of creams, gels, or drops, etc., the active compounds or their physiologically tolerated derivatives such as, salts, esters, or amides, are prepared and applied as solutions, suspensions, or emulsions in a physiologically acceptable diluent with or

5   without a pharmaceutical carrier.

[0035]   Another aspect of the present invention is directed to a method of treating a patient with tyrosinemia by administering to the patient an effective amount of a compound of formulas I or II or III or derivatives thereof as follows:

10   I   II   III

R^1, R^2, R^3, and R^4 each individually represent a hydrogen, an alkyl, alkenyl, or alkoxy group containing 1 to about 8 carbon atoms, an aryl, aralkyl, or cycloalkyl group containing about 5 to 12 carbon atoms, or a carboalkoxy or carbamyl group containing up to 8 carbon atoms, or a peptide or peptidomimetic moiety containing 10

15   to about 30 carbon atoms under conditions effective to treat tyrosinemia.

[0036]   This embodiment of the present invention can be carried out using the compounds and modes of administration described above.

[0037]   Yet another aspect of the present invention relates to a method of regulating plant growth by administering to a plant an effective amount of a

20   compound of formulas I or II or III or derivatives thereof as follows:

$R^1, R^2, R^3,$ and R^4 each individually represent a hydrogen, an alkyl, alkenyl, or alkoxy group containing 1 to about 8 carbon atoms, an aryl, aralkyl, or cycloalkyl group containing about 5 to 12 carbon atoms, or a carboalkoxy or carbamyl group

5   containing up to 8 carbon atoms, or a peptide or peptidomimetic moiety containing 10 to about 30 carbon atoms under conditions effective to regulate plant growth.

[0038]   This embodiment of the present invention can be carried out using the compounds described above. These compounds can be applied to plants or plant seeds, either by topical application such as spraying, or by systemic application via

10   absorption through the root system of a plant when delivered in solubilized form to said system.

[0039]   Suitable plants include dicots and monocots. More particularly, useful crop plants can include: alfalfa, rice, wheat, barley, rye, cotton, sunflower, peanut, corn, potato, sweet potato, bean, pea, chicory, lettuce, endive, cabbage, brussel sprout,

15   beet, parsnip, turnip, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, citrus, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane. Examples of suitable ornamental plants are: *Arabidopsis thaliana, Saintpaulia,* petunia, pelargonium, poinsettia, chrysanthemum, carnation,

20   and zinnia.

[0040]   The method of the present invention involving application of the compounds of the present application to plants can be carried out through a variety of procedures when all or part of the plant is treated, including leaves, stems, roots, etc. Suitable application methods include high or low pressure spraying, injection, and

25   leaf abrasion proximate to when application of the compound takes place. When treating plant seeds or propagules (e.g., cuttings), in accordance with the application

- 20 -

embodiment of the present invention, the compounds of the present invention can be applied by low or high pressure spraying, coating, immersion, or injection. Other suitable application procedures can be envisioned by those skilled in the art. Plants may be treated with one or more applications of the compounds of the present

5   invention to regulate plant growth.

[0041]   The compounds of the present invention, can be applied to plants or plant seeds alone or in a mixture with other materials. Alternatively, the compounds can be applied separately to plants with other materials being applied at different times.

10 [0042]   A composition suitable for treating plants or plant seeds in accordance with the application embodiment of the present invention contains the compound of the present invention in a carrier. Suitable carriers include water, aqueous solutions, slurries, or dry powders.

[0043]   Although not required, this composition may contain additional

15 additives including fertilizer, insecticide, fungicide, nematacide, and mixtures thereof. Suitable fertilizers include $(NH_4)_2NO_3$. An example of a suitable insecticide is Malathion. Useful fungicides include Captan.

[0044]   Other suitable additives include buffering agents, wetting agents, coating agents, and abrading agents. These materials can be used to facilitate the

20 process of the present invention. In addition, the compounds of the present invention can be applied to plant seeds with other conventional seed formulation and treatment materials, including clays and polysaccharides.

EXAMPLES

25

Example 1 - Experimental Design and Assays Used in the Clinical Investigation

[0045]   The investigation used IRB-approved protocols that form an integral part of the Children's Clinical Research Center (CCRC) at the Weill Medical College

30 of Cornell University. Measurements of the matrix biomarkers as well as the molecular modeling of 4-hydroxyphenylpyruvate and 2-(2-nitro-4-trifluoromethylbenzoyl)-cyclohexane-1,3-dione were conducted in the Connective Tissue Core Laboratory of the CCRC (Director: H.M. Hanauske-Abel, MD PhD).

[0046]   A total of ten biomarkers were monitored from just before initiation of
NTBC medication to up to 103 days of treatment, generating a total of 60 data points.
4-HPPD activity was assessed by excretion of succinylacetoacetate and
succinylacetone, metabolic derivatives of the 4-HPPD product homogentisic acid, and

5   by 4-aminolevulinate in urine. In plasma, methionine and succinylacetone, and in
serum α-fetoprotein also were quantified. These indices were determined by standard
methods (Lindstedt et al., "Treatment of Hereditary Tyrosinemia Type I by Inhibition
of 4-Hydroxyphenylpyruvate Dioxygenase," Lancet 340:813-17 (1992), which is
hereby incorporated by reference in its entirety). P4-H activity was assessed via

10   specific antigens released from three different groups of collagens, all of which are
substrates for this enzyme. These biomarkers, quantified by specific
radioimmunoassay, consisted of PIIINP, an indicator for the fibril-forming collagen
type III; NC1, a marker for the sheet-forming collagen type IV; and NTD, an indicator
for the beaded filament-forming collagen type VI. The P1 antigen, derived from the

15   non-collagenous protein laminin and thus not dependent on P4-H activity, served as
control.

Example 2 - Study Subject

20   [0047]   In an adopted child with unknown family history, tyrosinemia I was
diagnosed at age 4 months. Symptoms at presentation included: failure to thrive,
hepatosplenomegaly, rickets, coagulopathy, and Fanconi's syndrome. Because of the
coagulopathy, a liver biopsy was not performed at that time. The diagnosis of
tyrosinemia I was confirmed by the measurement of fumarylacetoacetate hydrolase

25   (EC 3.7.1.2) activity in blood and by the presence of abnormal levels of tyrosine and
tyrosine metabolites in serum and urine. Managed exclusively by nutritional therapy,
the patient developed along the 25th percentile for weight and the 5th percentile for
height, but experienced a tyrosinemic neurologic crisis at age 3 years, with severe
muscle weakness in the lower extremities and areflexia. Orfadin™ treatment

30   commenced at age 10 years at a dose of 10 mg po BID.

[0048]   Once tyrosinemia I was established biochemically, the need for a liver
biopsy was less pressing as the clinical status was assessed on the basis of liver
function parameters and, later, the response to NTBC. However, hepatic imaging

- 22 -

studies, which included magnetic resonance, ultrasound, and computerized tomography, uniformly document marked parenchymal inhomogeneity, reflective of fibrosis with regenerative nodules, or focal lesions in a cirrhotic liver (Rofsky et al., "CT and MRI of Diffuse Liver Disease," Semin. Ultrasound CT MR, 16:16-33

5   (1995), which is hereby incorporated by reference in its entirety). Portal hypertension has evolved and is evidenced by splenomegaly and hypersplenism, as well as by collateral circulation and a caput medusa pattern demonstrated by Doppler ultrasound (Kok et al., "The Value of Doppler Ultrasound in Cirrhosis and Portal Hypertension," Scand. J. Gastroenterol., 230(Suppl.):82-8 (1999), which is hereby incorporated by

10   reference in its entirety).

Example 3 - Computational Analysis of Molecules

[0049]   The sequences for 4-HPPD of *H. sapiens* (Accession #AAC73008) and

15   *P. fluorescens* (Accession #ICIXA) were aligned using the ClustalW algorithm of MacVector (Oxford Molecular, Madison,WI) to identify the active site segments and quantify their degree of conservation. The only publicly available crystal structure coordinates for 2-oxoacid-utilizing dioxygenases were downloaded from the PDB database under locator ICIX [4-HPPD of P. fluorescens] (Serre et al., "Crystal

20   Structure of *Pseudomonas* Fluorescens 4-Hydroxyphenylpyruvate Dioxygenase: An Enzyme Involved in the Tyrosine Degradation Pathway," Structure 7:977-88 (1999), which is hereby incorporated by reference in its entirety), and under locator IRXG [deactoxycephalosporin C synthase of *S. clavuligerus*] (Valegard et al., "Structure of a Cephalosporin Synthase," Nature 394:805-9 (1998), which is hereby incorporated by

25   reference in its entirety); the latter provides the representative coordinates for the alignment of the atmospheric oxygen and the 2-oxoacid substrates at the non-heme iron of these enzymes. Three-dimensional images of the active site of 4-HPPD were developed with the MSI InsightII software package (Molecular Simulations Inc., San Diego, CA). The 4-HPPD substrate 4-hydroxyphenylpyruvate (4-HPP) and the 4-

30   HPPD inhibitor 2-(2-nitro-4-trifluormethylbenzoyl)-cyclohexane-1,3-dione (NTBC) were modeled and optimized using MacSpartan Plus (Wavefunction Inc., Irvine, CA), and then exported as PDB files into MSI InsightII.

- 23 -

[0050]   It is well-established that the *in vivo* activity of the 2-oxoacid utilizing

dioxygenases 4-HPPD and P4-H, which both use the HAG mechanism for catalysis, is

directly reflected by their specific biomarkers. To assess 4-HPPD activity, two

deleterious metabolites of the enzyme's product homogentisic acid, i.e.,

5   succinylacetone and succinylacetoacetate, have been measured in biologic fluids

(Lindstedt et al., "Treatment of Hereditary Tyrosinemia Type I by Inhibition of 4-

Hydroxyphenylpyruvate Dioxygenase," Lancet 340:813-17 (1992); Holme et al.,

"Tyrosinemia Type I and NTBC [2-(2-Nitro-4-Trifluoromethylbenzoyl)-1,3-

Cyclohexanedione]," J. Inherit Metab. Dis., 21:507-17 (1998), which are hereby

10   incorporated by reference in their entirety). To assess P4-H activity, several collagen-

derived antigens have been employed. Since all collagen molecules depend on P4-H-

synthesized hydroxyproline residues to stabilize their triple helix (Bella et al.,

"Crystal and Molecular Structure of a Collagen-Like Peptide at 1.9Å Resolution,"

Science 266:75-81 (1994), which is hereby incorporated by reference in its entirety),

15   inhibition of P4-H renders helix formation impossible (Hanauske-Abel, "Fibrosis:

Representative Molecular Elements, A Basic Concept, and Emerging Targets for

Suppressive Treatment," in Zakim, eds. Hepatology, A Textbook of Liver Disease,"

Philadelphia: W.B. Saunders, pp. 465-506 (1996); Hanauske-Abel, "Prolyl 4-

Hydroxylase, A Target Enzyme for Drug Development. Design of Suppressive

20   Agents and the In Vitro Effects of Inhibitors and Proinhibitors," J. Hepatol.,

13(Suppl.3):S8-S16 (1991), which are hereby incorporated by reference in their

entirety). In the case of fibrillar collagens like type III, the underhydroxylated non-

helical material is retained and degraded intracellularly (Tschank et al., "Pyridine-

Dicarboxylates, the First Mechanism-Derived Inhibitors for Prolyl 4-Hydroxylase,

25   Selectively Suppress Cellular Hydroxyproline Biosynthesis," Biochem J. 248:625-33

(1987), which is hereby incorporated by reference in its entirety). P4-H inhibition in

cell culture (McCaffrey et al., "Specific Inhibition of eIF-5A and Collagen

Hydroxylation by a Single Agent: Antiproliferative and Fibro-Suppressive Effects on

Smooth Muscle Cells from Human Coronary Arteries," J. Clin. Invest. 95:446-55

30   (1995), which is hereby incorporated by reference in its entirety) or in patients

(Hanauske-Abel, "Fibrosis: Representative Molecular Elements, A Basic Concept,

and Emerging Targets for Suppressive Treatment," in Zakim, eds. Hepatology, A

Textbook of Liver Disease," Philadelphia: W.B. Saunders, pp. 465-506 (1996);

Newfield et al., "Deferiprone, but not Deferoxamine, Inhibits Biosynthesis of Fibrillar

Procollagen in Iron-Overloaded Thalassemia Patients," Pediatr. Res. 39:A943 (1996),

which are hereby incorporated by reference in their entirety) causes a marked and

5   rapid decrease in collagen biomarkers levels. In the case of sheet-forming collagens

like type IV, the under-hydroxylated non-helical material may be secreted, whereupon

it fails to incorporate into matrix and is degraded extracellularly (Kim et al.,

"Differential Effects of Ascorbate Depletion and Alpha, Alpha-Dipyridyl Treatment

on the Stability, but not on the Secretion, of Collagen Type IV Collagen in

10   Differentiated P9 Cells," J. Cell Biochem. 67:338-52 (1997); Frei et al., "The N-

Terminal Propeptide of Collagen Type III in Serum Reflects Activity and Degree of

Fibrosis in Patients with Chronic Liver Disease," Hepatology 4:830-834 (1984),

which are hereby incorporated by reference in their entirety). These collagen type-

specific consequences of P4-H inhibition suggested the use of several, collagen-type

15   specific biomarkers, i.e., of NCI and NTD in addition to PIIINP. The serum

concentration of each of these biomarkers, derived from collagens type III, type IV,

and type VI, respectively, also is known to be directly related to, and markedly

increases with, fibrotic liver disease (Murawaki et al., "Comparison of Serum 7S

Fragment of Type IV Collagen and Serum Central Triple-Helix of Type IV Collagen

20   for Assessment of Liver Fibrosis in Patients with Chronic Viral Liver Disease," J.

Hepatol. 24:148-54 (1996); Pitkanen et al., "Serum Type III Procollagen in Children

with Type I Hereditary Tyrosinemia," J. Ped. Gastroenterol. Nutr. 29:38-41 (1999),

which are hereby incorporated by reference in their entirety). The P1 antigen of

laminin, the major matrix protein that lacks hydroxyproline residues and in culture is

25   secreted normally even as P4-H is completely inhibited (Kim et al., "Differential

Effects of Ascorbate Depletion and Alpha, Alpha-Dipyridyl Treatment on the

Stability, but not on the Secretion, of Collagen Type IV Collagen in Differentiated P9

Cells," J. Cell Biochem. 67:338-52 (1997), which is hereby incorporated by reference

in its entirety), was measured as control parameter.

30   [0051]   In the tyrosinemic patient, the biomarkers for 4-HPPD activity

promptly decreased upon initiation of NTBC treatment. Initially elevated to more

than ten-fold its physiological level, the excretion of succinylacetoacetate normalized

within two weeks. The grossly increased plasma concentration of succinylacetone also fell, although more gradually, and stabilized in the normal range after two months of treatment (Figure 1). These observations are consistent with the previously reported effect of NTBC on these biomarkers for the activity of human 4-HPPD, and

5   confirm the distinctly different kinetics of their response (Lindstedt et al., "Treatment of Hereditary Tyrosinemia Type I by Inhibition of 4-Hydroxyphenylpyruvate Dioxygenase," Lancet 340:813-17 (1992), which is hereby incorporated by reference in its entirety).

[0052]   The biomarkers for P4-H activity were all entirely unresponsive to

10   NTBC, however (Figure 1). The levels of PIIINP, NC1, and NTD, documented before initiation of treatment, ranged at the minimum 1.6 multiples above the age-related medians, and as such revealed an active and on-going fibrogenic process in the patient's liver (Murawaki et al., "Comparison of Serum 7S Fragment of Type IV Collagen and Serum Central Triple-Helix of Type IV Collagen for Assessment of

15   Liver Fibrosis in Patients with Chronic Viral Liver Disease," J. Hepatol. 24:148-54 (1996); Pitkanen et al., "Serum Type III Procollagen in Children with Type I Hereditary Tyrosinemia," J. Ped. Gastroenterol. Nutr. 29:38-41 (1999), which are hereby incorporated by reference in their entirety). These initially abnormal levels remained consistently elevated throughout the three months of the study period and

20   were unaffected by the administration of NTBC, although in humans, effective P4-H inhibition causes the biomarkers for at least the fibrillar collagens to decrease within a few days (Hanauske-Abel, "Fibrosis: Representative Molecular Elements, A Basic Concept, and Emerging Targets for Suppressive Treatment," in Zakim, eds. Hepatology, A Textbook of Liver Disease," Philadelphia: W.B. Saunders, pp. 465-

25   506 (1996); Newfield et al., "Deferiprone, but not Deferoxamine, Inhibits Biosynthesis of Fibrillar Procollagen in Iron-Overloaded Thalassemia Patients," Pediatr. Res. 39:A943 (1996), which are hereby incorporated by reference in their entirety). In fact, all the biomarkers for collagenous and, therefore, P4-H-dependent proteins (i.e., PIIINP, NC1, and NTD) were consistently just as unresponsive to

30   NTBC treatment as the non-collagenous and, therefore, P4-H independent protein laminin, assessed via its P1 antigen (Figure 1). After 103 days on NTBC, all the biomarkers for 4-HPPD activity had normalized, averaging 3% of their initial values,

- 26 -

whereas the PIIINP biomarker for P4-H activity displayed a distinct increase to 1.25 times its pre-NTBC level, or three times the upper limit of normal. The other P4-H biomarkers also did not just fail to decrease, but showed a similar, though smaller rise over their initial, abnormally elevated values, NCI and NTC both increasing by a

5   factor of 1.1. After 273 days on NTBC, PIIINP had increased even further, to 1.35 times its value at treatment initiation.

[0053]   While applicants' investigation was in progress, a brief report described in one other tyrosinemic child that the initially elevated PIIINP level was non-responsive to treatment with NTBC (Pitkanen et al., "Serum Type III Procollagen

10   in Children with Type I Hereditary Tyrosinemia," J. Ped. Gastroenterol. Nutr. 29:38-41 (1999), which is hereby incorporated by reference in its entirety). This case observation and the applicants' results, summarized in Figure 1, indicate that *in vivo*, NTBC affects the 2-oxoacid utilizing dioxygenases 4-HPPD and P4-H in an entirely different manner, all the biomarkers for the former being highly susceptible, and all

15   those for the latter totally resistant to inhibition. Using an unspecified *in vitro* assay for P4-H activity isolated from an unidentified species, the developers of NTBC cursorily mentioned a similar observation (Ellis et al., "Inhibition of 4-Hydroxyphenylpyruvate Dioxygenase by 2-(2-Nitro-4-Trifluormethylbenzoyl)-Cyclohexane-1,3-Dione and by 2-(2-Chloro-4-Methanesulfonylbenzoyl)-

20   Cyclohexane-1,3-Dione," Toxicol. Appl. Pharmacol. 133:12-19 (1995), which is hereby incorporated by reference in its entirety). Together, these repeatedly noted differences indicate that the inhibitor NTBC only affects the toxogenic activity of 4-HPPD in tyrosinemic patients, but does not affect the fibrogenic activity of prolyl 4-hydroxylase, although both enzymes belong to the same class of dioxygenases and

25   follow the same catalytic mechanism. The patients thus remain vulnerable to long-term liver failure due to the slowly progressing fibrosis and evolving cirrhosis. By contrast, compounds of the present invention as embodied by DMHP are known inhibitors of prolyl 4-hydroxylase and, therefore, collagen biosynthesis (United States Patent Nos. 5,789,426; 5,965,585; and 5,965,586, which are hereby incorporated by

30   reference in their entirety), and also fit the active site of 4-HPPD, thus blocking the enzyme's toxogenic activity in these patients in a manner similar to NTBC (see Figure 3).

[0054]   The stereochemistry of substrate and inhibitor binding to P4-H is

known (Hanauske-Abel, "Prolyl 4-Hydroxylase, A Target Enzyme for Drug

Development. Design of Suppressive Agents and the In Vitro Effects of Inhibitors

and Proinhibitors," J. Hepatol., 13(Suppl.3):S8-S16 (1991), which is hereby

5   incorporated by reference in its entirety). The stereochemistry of substrate and

inhibitor binding to 4-HPPD, though highly relevant for the current state-of-the-art

treatment of tyrosinemia I, has not been formulated, yet it is evident from

crystallographic and computational data, as summarized in Figure 2. Consistent with

the HAG mechanism (Hanauske-Abel et al., "A Stereochemical Concept for the

10   Catalytic Mechanism of Prolyl Hydroxylase. Applicability to Classification and

Design of Inhibitors," J. Theor. Biol. 94:421-55 (1982); Hanauske-Abel, "Fibrosis:

Representative Molecular Elements, A Basic Concept, and Emerging Targets for

Suppressive Treatment," in Zakim, eds. Hepatology, A Textbook of Liver Disease,"

Philadelphia: W.B. Saunders, pp. 465-506 (1996), which are hereby incorporated by

15   reference in their entirety), the enzyme's substrate 4-hydroxyphenylpyruvate (4-HPP)

displays two domains, one defined by the benzene ring (domain I) and one by the 2-

oxoacid group (domain II); the same domain structure applies to NTBC (Figure 2A).

In both agents, these domains define planes that are markedly out-of-level with each

other (Figure 2B). In the case of 4-HPP, domain I is rotated by almost 70° above

20   domain II and oriented towards the binding site of dioxygen, the source of the reactive

oxygen atom that, in the product-forming step, transfers from the iron onto the

benzene ring (Abbott et al., "Alpha-Ketoglutarate-Coupled Dioxygenases," in

Hayaishi, ed. Molecular Mechanisms of Oxygen Activation, New York: Academic

Press, pp. 167-214 (1974); Hanauske-Abel et al., "A Stereochemical Concept for the

25   Catalytic Mechanism of Prolyl Hydroxylase. Applicability to Classification and

Design of Inhibitors," J. Theor. Biol. 94:421-55 (1982), which are hereby

incorporated by reference in their entirety). In the case of NTBC, domain I also is

oriented towards that same binding site, which places the electronegative nitro group

precisely into the position usually occupied by the electronegative dioxygen

30   (Figure 2B). This computer-generated alignment suggests that the ortho-positioned

nitro group can, in a dioxygen-like manner, directly interact with the active site iron,

an effect that at the active site of the enzyme would make NTBC a terdentate ligand,

in contrast to the bidentate coordination of 4-HPP (Figure 2). Indeed, experimental

studies of the structure-activity relation for NTBC and similar cyclohexanetrionics

confirm that the presence of an ortho-positioned electronegative group, carried by a

properly oriented benzene ring, is important for the effective inhibition of 4-HPPD

5   (Lin et al. "SAR Studies of 2-O-substituted-benzoyl- and 2-alkanoyl-cyclohexane-1,3-

diones as Inhibitors of 4-hydroxyphenylpyruvate dioxygenases," Bioorg. Med. Chem.

Lett. 10:843-45 (2000), which is hereby incorporated by reference in its entirety). In

applicants' analysis, the ability to coordinate to, and thus block, the catalytically

essential non-heme iron atom emerges as an important feature of NTBC binding to

10   the active site of 4-HPPD. This pivotal conclusion was subsequently corroborated by

independent investigators (Wu et al. "Mode of Action of 4-hydroxyphenylpyruvate

dioxygenase Inhibition by Triketone-type Inhibitors," J. Med. Chem. 45:2222-28

(2002), which is hereby incorporated by reference in its entirety). This ability is a

general characteristic of effective inhibitors for other 2-oxoacid-utilizing

15   dioxygenases, in particular P4-H (Hanauske-Abel, "Prolyl 4-Hydroxylase, A Target

Enzyme for Drug Development. Design of Suppressive Agents and the In Vitro

Effects of Inhibitors and Proinhibitors," J. Hepatol., 13(Suppl.3):S8-S16 (1991);

Günzler et al., "Prolyl 4-Hydroxylase Inhibitors," in Guzman NA ed., Prolyl

Hyroxylase, Protein Disulfide Isomerase, and Other Structurally Related Proteins,

20   New York: Decker, pp. 65-96 (1997); Ng et al., "The Cosubstrate Binding Site of

Pseudomonas sp. AK1 γ-Butyrobetaine Hydroxylase: Interactions with Structural

Analogs of α-Ketoglutarate," J. Biol. Chem. 266:1526-33 (1991), which are hereby

incorporated by reference in their entirety). However, the active site of P4-H requires

in addition to this general characteristic, that domain I is positioned in the plane of

25   domain II, rendering P4-H particularly susceptible to entirely planar inhibitors

(Hanauske-Abel, "Über einen stereochemischen Vorschlag für den katalytischen

Mechanismus der Prolylhydroxylase, seine Anwendung zur Klassifizierung und

Formulierung von Hemmstoffen sowie die Entwicklung und Erprobung eines

'maßgeschneiderten' neuartigen Inhibitors," Department of Pharmacol. Toxicol.,

30   Philipps University, Marburg an der Lahn, Germany, M.D., Ph.D., Thesis (1983);

Günzler et al., "Prolyl 4-Hydroxylase Inhibitors," in Guzman NA ed., Prolyl

Hyroxylase, Protein Disulfide Isomerase, and Other Structurally Related Proteins,

New York: Decker, pp. 65-96 (1997); Ng et al., "The Cosubstrate Binding Site of

Pseudomonas sp. AK1 γ-Butyrobetaine Hydroxylase: Interactions with Structural

Analogs of α-Ketoglutarate," J. Biol. Chem. 266:1526-33 (1991), which are hereby

incorporated by reference in their entirety). The out-of-level alignment of domain I-

5   vs.-domain II, as visualized in Figure 2 for 4-HPPD, is in distinct contrast to the

conformation of all known inhibitors for P4-H. 4-HPPD and P4-H should, therefore,

display markedly different susceptibilities to NTBC. As shown in Figure 1, this is

indeed the case. In contrast to NTBC, the compounds of the present invention as

embodied by DMHP lack any structural equivalent of domain I (compare Figure 2B

10   with Figure 3) and therefore, are not subject to the biological and clinical

consequences of misalignment between these domains. In fact, DMHP so closely

mimics the dimensions and the planarity of domain II of the 4-HPP substrate that both

entities assume isomorphic positioning at the active site of 4-HPPD, as shown in

Figure 3. The structure-based approach of the present invention also is applicable to

15   the evaluation of agents other than DMHP or NTBC. For instance, usnic acid, a

metabolite produced by lichen to suppress competing plants (Moradpur et al.,

"Hepatic Oncogenesis," in Zakim, eds., Hepatology, Philadelphia: Saunders,

pp. 1490-1512 (1996), which is hereby incorporated by reference in its entirety) and

an ingredient in over-the-counter dietary supplements, is a potent inhibitor of plant 4-

20   HPPD (Romagni et al., "The Phytotoxic Lichen Metabolite, Usnic Acid, is a Potent

Inhibitor of Plant p-hydroxyphenylpyruvate dioxygenase," FEBS Lett. 480:301-05

(2000), which is hereby incorporated by reference in its entirety) and fits the 4-HPPD

model presented in Figure 2. This provides a rationale for the clinical evaluation of

usnic acid as a 4-HPPD inhibitor in tyrosinemia I. In general, computer-assisted

25   modeling has been extraordinarily successful in guiding the development of inhibitors

for several enzymes in the class of 2-oxoacid utilizing dioxygenases (Hanauske-Abel

et al., "The HAG Mechanism: A Molecular Rationale for the Therapeutic Application

of Iron Chelators in Human Diseases Involving the 2-oxoacid Utilizing

Dioxygenases," Curr. Med. Chem. 10:1005-19 (2003), which is hereby incorporated

30   by reference in its entirety).

[0055]   The persistent elevation of several indicators for hepatic fibrogenesis

suggests that even after NTBC medication achieves biochemical normalization of the

toxic metabolites of 4-HPPD in tyrosinemia I, the biologic correction of the fibrotic process does not occur readily (Figure 1; also Pitkanen et al., "Serum Type III Procollagen in Children with Type I Hereditary Tyrosinemia," J. Ped. Gastroenterol. Nutr. 29:38-41 (1999), which is hereby incorporated by reference in its entirety). This

5   caveat is supported by the histopathologic evaluation of repeated liver biopsies performed on patients with tyrosinemia I before and during prolonged treatment with NTBC. The abnormal hepatic metabolism of several major collagens, sustained at a markedly increased level, should raise the concerns that over the lifetime of these children, a fibrotic process even if low-grade may eventually lead to cirrhosis and

10   long-term organ failure, and may figure as one factor in the development of hepatocellular carcinoma. The latter, which occurs in 40% of tyrosinemic patients not treated with NTBC (Moradpur et al., "Hepatic Oncogenesis" In Hepatology. D. Zakim and T.D. Boyer, ed. Saunders, Philadelphia. 1490-1512 (1996), which is hereby incorporated by reference in its entirety), has been reported during the first

15   year (Holme et al., "Nontransplant Treatment of Tyrosinemia," Clin. Liver Dis. 4:805-814 (2000), which is hereby incorporated by reference in its entirety) and the second year (Viladoms et al., "Evolution of Tyrosinemia Type I Treated with NTBC," An Esp. Pediatr. 54:305-9 (2001), which is hereby incorporated by reference in its entirety) of treatment with NTBC. While early treatment with NTBC may decrease

20   the incidence of this serious complication (Holme et al., "Tyrosinemia Type I and NTBC [2-(2-Nitro-4-Trifluoromethylbenzoyl)-1,3-Cyclohexanedione]," J. Inherit Metab. Dis., 21:507-17 (1998); Russo et al., "Tyrosinemia: A Review," Pediatr. Dev. Pathol. 4:212-221 (2001), which are hereby incorporated by reference in their entirety), it remains entirely unknown at present whether treatment with NTBC affects

25   the evolving liver fibrosis and its sequelae, such as portal hypertension, which like hepatocellular carcinoma rank among the major issues in the care for these children (Russo et al., "Tyrosinemia: A Review," Pediatr. Dev. Pathol. 4:212-221 (2001), which is hereby incorporated by reference in its entirety). By contrast, in experimental animals, the targeted inhibition of P4-H, which markedly suppresses

30   hepatic fibrosis, limits both the development of portal hypertension (Hanauske-Abel, "Fibrosis: Representative Molecular Elements, A Basic Concept, and Emerging Targets for Suppressive Treatment," in Zakim, eds. Hepatology, A Textbook of Liver

- 31 -

<u>Disease,</u>" Philadelphia: W.B. Saunders, pp. 465-506 (1996), which is hereby

incorporated by reference in its entirety) and of hepatic neoplasms (Sakaida et al.,

"Prevention of Fibrosis Reduces Enzyme-altered Lesions in the Rat Liver,"

<u>Carcinogenesis</u> 15:2201-06 (1994), which is hereby incorporated by reference in its

5   entirety). Joint inhibition of 4-HPPD and P4-H, or inhibition of just P4-H, would be

particularly reassuring for these patients, and would also provide an invaluable inroad

into the development of P4-H inhibitors for use as hepatoselective fibrosuppressors

(Hanauske-Abel, "Fibrosis: Representative Molecular Elements, A Basic Concept,

and Emerging Targets for Suppressive Treatment," in Zakim, eds. <u>Hepatology, A</u>

10   <u>Textbook of Liver Disease,</u>" Philadelphia: W.B. Saunders, pp. 465-506 (1996);

Hanauske-Abel, "Prolyl 4-Hydroxylase, A Target Enzyme for Drug Development.

Design of Suppressive Agents and the In Vitro Effects of Inhibitors and

Proinhibitors," <u>J. Hepatol.,</u> 13(Suppl.3):S8-S16 (1991), which are hereby incorporated

by reference in their entirety), compounds of general interest in treatment of fibrotic

15   liver disease. Applicants' computational analysis of agents that already are clinically

available, suggests that hydroxypyridone compounds not only inhibit P4-H (U.S.

Patent No. 5,965,586 to Hanauske-Abel et al. and Clement et al., "The Antifungal

Drug Ciclopirox Inhibits Deoxyhypusine and Proline Hydroxylation, Endothelial Cell

Growth, and Angiogenesis In vitro," <u>Int'l J. Cancer</u> 100:491-98 (2002), which are

20   hereby incorporated by reference in their entirety) and selectively suppress collagen

formation (U.S. Patent No. 5,789,426 to Hanauske-Abel et al., which is hereby

incorporated by reference in its entirety), but also fit the active site of 4-HPPD. As

exemplified in Figure 3 by DMHP, hydroxypyridones locate precisely into the van der

Waals volume occupied at the active site by the cyclohexane-1,3-dione moiety of

25   NTBC and its analogs. It is, therefore, evident that the hydroxypyridone inhibitors of

P4-H will also inhibit 4-HPPD *in vitro* and *in vivo*, will be useful as pilot structures

for chemical optimization, and represent promising candidates for clinical trials.

[0056]   Finally, in addition to the 3D coordinate of 4-HPPD, the crystal

structures of several additional 2-oxoacid-utilizing dioxygenases have been deposited

30   in the PDB database (Berman et al. "The Protein Data Bank" <u>Nucl. Acids Res.</u>

28:235-42 (2000), which is hereby incorporated by reference in its entirety). Those

coordinates for 4-HPPD are at locator 1CJX and have been used by the applicants.

- 32 -

Currently, crystal structures are available for the following enzymes that also employ
the HAG mechanism: clavaminate synthase (locators 1DS0, 1DS1, 1DRT, 1DRY);
carbapenem synthase (locators 1NX8, 1NX4); deactoxycephalosporin C synthase
(locators 1RXG, 1RXF, 1E5I, 1E5H); anthocyanidin synthase (locators 1GP4, 1GP5,
5   1GP6); proline 3-hydroxylase (locator 1E5R, 1E5S); taurine dioxygenase (1GY9,
1GQW); and the first representative example for the sub-family of protein
hydroxylases, FIH1, one of the enzymes which modify and control hypoxia inducible
factor-1 alpha (locators 1H2K, 1H2L, 1H2M, 1H2N, 1MZF). The active site
coordinates of all these enzymes reveal the same cisoid coordination of a non-heme
10   ferrous ion, with the same relative orientation of two histidines and one carboxyl side
chain (compare Figures 2 and 3) imposed by the HAG mechanism (Hanauske-Abel et
al., "The HAG Mechanism: A Molecular Rationale for the Therapeutic Application of
Iron Chelators in Human Diseases Involving the 2-Oxoacid Utilizing Dioxygenases,"
Curr. Med. Chem. 10:1005-19 (2003), which is hereby incorporated by reference in
15   its entirety). This atomic arrangement at the active site of all enzymes of this class,
summarized in Table III, also provides the coordination sites at the catalytic ferrous
ion that are required for interaction with, and inhibition by, the hydroxypyridone
compounds of the present invention.

Table III : Representative examples for HAG dioxygenases and their biological functions.*

Enzyme	Dependent biological event	Species
Prolyl 4-hydroxylase (I and II)	Synthesis of collagens and collectins	*H, A*
HIF Prolyl 4-hydroxylase (PHD1 - 3)	Oxygen-sensing via hypoxia-inducible factor	*H, A*
Aspartyl/asparaginyl 3-hydroxylase	Synthesis of fibrillins and LTBPs Bioactivity of TGF-ßs	*H, A*
HIF Aspartyl/asparaginyl 3-hydroxylase (FIH-1)	Oxygen-sensing via hypoxia-inducible factor	*H*
Deoxyhypusyl hydroxylase	Control of G1-to-S transition in the cell cycle, i.e. initiation of DNA replication; Multiplication of retroviruses, e.g. HIV-1	*H, A, Pl, fungi and archaea*
Lysyl 5-hydroxylase 1 to 3	Crosslinking / glycosylation of collagens	*H, A*
4-Hydroxyphenylpyruvate dioxygenase *apparently identical with*	Tyrosine catabolism Tyrosine catabolism, antioxidant synthesis Plastochinone / tocopherol synthesis	*H, A, M Pl*
α-Ketoisocaproate oxygenase	Leucine catabolism	*H, A*
AlkB protein	Reversal of DNA alkylation	*H M*
ε-N-Trimethyllysine hydroxylase	Carnitine formation	*H, A, M*
γ-Butyrobetaine hydroxylase	Carnitine formation	*H, A, M*
Phytanoyl-CoA 2-hydroxylase	Phytanic acid metabolism	*H*
Hyoscyamine 6β-hydroxylase	Atropine / scopolamine synthesis	*Pl*
Desacetoxyvindoline 4-hydroxylase	Vincristine / vinblastine synthesis	*Pl*
Anthocyanidin synthase	Anthocyanin and related plant colors	*Pl*
2,4-dichlorophenoxyacetic acid dioxygenase	Herbicide metabolism	*Pl*
Isoleucine 4-hydroxylase	Unknown	*Pl*
Flavone synthase I	Flavone synthesis, nodulation	*Pl*
(2S)-Flavanone 3ß-hydroxylase	Catechin / anthocyanidin synthesis	*Pl*
Flavanol 6-hydroxylase	Flavanol synthesis	*Pl*
Gibberellin 2β-hydroxylase	Plant growth	*Pl*
Gibberellin 3β-hydroxylase	Plant growth	*Pl*
Gibberellin 20-hydroxylase	Plant growth	*Pl*
Prolyl 4-hydroxylase	Cell wall synthesis, defense	*Pl*
Proline 4-hydroxylase	Actinomycin synthesis	*M*
Proline 3-hydroxylase	3-hydroxyproline formation	*M*
Thymidine 2'-hydroxylase	Nucleotide salvage	*M*
Thymine 7-hydroxylase	Thymine catabolism	*M*
Taurine dioxygenase	Sulfur metabolism	*M*
2-hydroxymyristate dioxygenase	Lipid A / endotoxin synthesis	*M*
Deactoxycephalosporin C synthase	Cephalosporin synthesis	*M*
Deactoxycephalosporin C hydroxylase (3'-methylcephem hydroxylase)	Cephalosporin synthesis	*M*
Deactoxycephalosporin C synthase/hydroxylase	Cephalosporin synthesis	*M*
Cephalosporin 7α-hydroxylase	Cephamycin synthesis	*M*
Clavaminate synthase	ß-Lactamase inhibitor synthesis	*M*

5

Abbreviations : Humans (H); animals (A); plants (Pl): microorganisms (M)

10

- 34 -

[0057]   Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.